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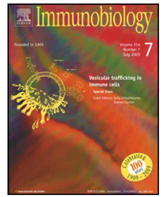

IL-27 enhances *Leishmania amazonensis* infection via ds-RNA dependent kinase (PKR) and IL-10 signaling

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ABSTRACT

The protozoan parasite *Leishmania* infects and replicates in macrophages, causing a spectrum of diseases in the human host, varying from cutaneous to visceral clinical forms. It is known that cytokines modulate the immunological response against *Leishmania* and are relevant for infection resolution. Here, we report that Interleukin (IL)-27 increases *Leishmania amazonensis* replication in human and murine macrophages and that the blockage of the IL-10 receptor on the surface of infected cells abolished the IL-27-mediated enhancement of *Leishmania* growth. IL-27 induced the activation/phosphorylation of protein kinase R (PKR) in macrophages, and PKR blockage or PKR gene deletion abrogated the enhancement of the parasite growth driven by IL-27, as well as the *L. amazonensis*-induced macrophage production of IL-27. We also observed that *L. amazonensis*-induced expression of IL-27 depends on type I interferon signaling and the engagement of Toll-like receptor 2. Treatment of *Leishmania*-infected mice with IL-27 increased lesion size and parasite loads in the footpad and lymph nodes of infected animals, indicating that this cytokine exerts a local and a systemic effect on parasite growth and propagation. In conclusion, we show that IL-27 is a *L. amazonensis*-enhancing factor and that the PKR/IFN1 axis and IL-10 are critical mediators of this IL-27 induced effect.

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Introduction

Leishmania is a kinetoplastid protozoan parasite that undergoes its life cycle in both invertebrate and vertebrate hosts (Kaye and Scott, 2011). Once in macrophages, the parasite differentiates into amastigote forms and extensively proliferates inside the intracellular phagolysosome, eventually lysing the infected cells and subsequently invading neighboring macrophages (Kaye and Scott, 2011). *Leishmania spp.* infections can cause a spectrum of clinical forms in the human host, varying from cutaneous lesions to visceral diseases (Kaye and Scott, 2011). It is estimated that leishmaniasis affects approximately 12 million people worldwide (World Health Organization, 2010).

It has been reported that cytokines play an important role in the control of *Leishmania* growth in macrophages (Sacks and Noben-Trauth, 2002), modulating the immunological response against the parasite and influencing infection resolution (Sacks and Noben-Trauth, 2002). Studies have shown that an immune response with a predominating production of T helper (Th)-2 cytokines contributes to replication of *Leishmania* (Sacks and Noben-Trauth, 2002), whereas, in contrast, the development of a T helper (Th) 1 response is detrimental to *Leishmania* survival and replication within macrophages. The presence and combination of cytokines and the moment and the context that they bind to cells

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(e.g., during antigen presentation) also influence the overall response against *Leishmania*. To date, a plethora of cytokines has been described to influence the establishment or replication of *Leishmania* (Kaye and Scott, 2011; Sacks and Noben-Trauth, 2002; Cummings et al., 2010; Locksley et al., 1987; Kurtzhals et al., 1994). In this study, we show that Interleukin (IL)-27 directly promotes the replication of *Leishmania amazonensis* in both human and murine macrophages, thus unveiling a novel biological function for this cytokine in the context of the biology of the infection by protozoan parasites.

The biologically active form of IL-27 is a heterodimeric cytokine structurally related to IL-12 and is constituted by the Epstein–Barr virus-induced gene 3 (EBI-3) and protein 28 (p28) subunits. The IL-27 receptor (IL-27R) is heterodimeric as well, formed by the WSX-1 (also called IL-27 receptor α chain) and glycoprotein 130 (gp130) molecules (Yoshida and Miyazaki, 2008; Yoshida et al., 2009). IL-27 is produced mostly by dendritic cells and macrophages following different stimuli, such as Toll-like receptor (TLR) ligands, statins or type 1 Interferons (IFN1) (Wirtz et al., 2005; Hause et al., 2007; Molle et al., 2007; Iyer et al., 2010). IL-27 regulates IL-10 production (Iyer et al., 2010; Batten et al., 2008; Ansari et al., 2011; Sun et al., 2011; Wang et al., 2011) and significantly contributes to suppress inflammatory processes, as recently reported in the immunopathology of severe malaria (Freitas do Rosário et al., 2012). It has been shown that IL-27 presents a relevant role in the early development of the Th1 response (Pflanz et al., 2002), thus, deficiency of IL-27 or its receptor can favor the growth and establishment of parasitic infections in which an initial Th1 response is detrimental to parasites, such as *Toxoplasma gondii*, *Trypanosoma cruzi*, *Leishmania major* and *Leishmania donovani* (Hunter et al., 2004). On the other hand, mice genetically deficient of the IL-27R subunit WSX-1 develop an early Th2 response that prevents gut colonization by *Trichuris muris*, which promotes a prompt expulsion of the worms (Bancroft et al., 2004). IL-27 is also endowed with potent inhibitory effect against human immunodeficiency virus type-1 (HIV-1) (Greenwell-Wild et al., 2009).

The IFN-induced double-stranded RNA-activated protein kinase (PKR) contains two functionally distinct domains, including a regulatory amino-terminal domain that consists of two double-stranded RNA-binding sites, and a catalytic carboxy-terminal domain that mediates serine and threonine phosphorylation (Meurs et al., 1990; Sadler and Williams, 2007). PKR is constitutively expressed in lymphocytes and macrophages, and its activation can occur after cellular exposure to different stimuli and stresses, such as double-stranded viral RNAs, dextran sulfate, chondroitin sulfate, heparin, growth factors and cytokines (Sadler and Williams, 2007). Upon activation, PKR phosphorylates the serine 51 located at the alpha subunit of eukaryotic initiation factor 2α (eIF2α), which leads to protein synthesis inhibition. A cascade of signaling events that modulate the activity of other proteins also occurs upon PKR activation, including the expression of genes involved in immune responses (e.g., IFN-β and IL-10) (Sadler and Williams, 2007; Raven and Koromilas, 2008). It has been reported that IL-27 initiates the transcription of multiple IFN-induced genes in macrophages and T cells, including the PKR/EIF2AK gene (Imamichi et al., 2008, 2012; Silva et al., 2004). We recently described that PKR activation favors the intracellular growth of *L. amazonensis*, due to parasite-induced IL-10 expression (Pereira et al., 2010), and we also showed the pivotal importance of the PKR/IFN1 axis and the engagement of TLR2 in *L. amazonensis* growth in macrophages (Vivarini et al., 2011). Taking into account the evidence of a close association between IL-27, IL-10, PKR and *Leishmania*, we investigated whether IL-27 could induce PKR activation and modulate *L. amazonensis* infection. In this work, we report, for the first time, that IL-27 promotes the growth of *L. amazonensis* through a signaling pathway that encompasses PKR/IFN1 axis and IL-10 signaling.

Materials and methods

Human primary macrophages and murine RAW 264.7 cells

Primary human monocyte-derived macrophages were obtained from peripheral blood mononuclear cells (PBMCs) isolated by density gradient centrifugation (GE Healthcare) of buffy coat preparations from healthy blood donors, as described elsewhere (Barreto-de-Souza et al., 2006). In brief, PBMCs were suspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% human serum (HS; Millipore) and then plated in permanox chambers slides (Lab-Tek®, Nalge Nunc; 8×10^5 cells/500 μL of medium) and incubated at 37 °C, 5% CO_2 for one week. Then, the non-adherent cells were washed out, fresh medium was added back, and the mature macrophage monolayer was maintained in culture for two more days at the same conditions mentioned above. The macrophage purity was ≥95% as determined by flow cytometry (BD FACS Canto II) with the use of labeled anti-CD68 antibodies (Santa Cruz Biotechnology). All experimental procedures involving human cells were approved by the Oswaldo Cruz Foundation/Fiocruz Research Ethics Committee (Rio de Janeiro, RJ, Brazil), under the number 397-07.

RAW 264.7 cells transfected with either empty (RAW-bla) or PKR K296R (RAW-DN-PKR) containing plasmids were generated as described previously (Imamichi et al., 2008) and maintained in RPMI medium supplemented with 10% fetal calf serum (FCS; Hyclone) and 1 μg/mL basticidin for plasmid selection. Twenty-four hours before experimentation, the cells were detached from culture flasks and seeded into chamber slide wells (Lab-tek®, NalgeNunc International) at 3×10^4 cells/well.

Murine peritoneal macrophages

Ten-week-old male 129/SvEv PKR$^{-/-}$ (PKR KO) mice and their respective wild type littermates (WT) were used for experiments involving PKR. Briefly, four days before peritoneal lavage, 2 mL of 3% thioglycolate were intra-peritoneally injected in each mouse. Peritoneal macrophages were harvested after 10 mL injection of cold DMEM, and total peritoneal cells were suspended in DMEM supplemented with 10% FCS and plated into chamber slides at 3×10^5 cells/well. Following overnight incubation, non-adherent cells were removed after a wash with warmed PBS. For assays related to IFN1 and TLR2, 10-week-old-male 129/SvEv (IFNR1-KO) and C57BL/6 (TLR2-KO) mice and their respective wild type littermates were used according to the same protocol described above. The experimental protocols using mice were approved by the Federal University of Rio de Janeiro Committee for Animal Use (permit numbers: IMPPG 024 and IBCCF171).

Parasites and macrophage infection

Promatigote forms of *L. amazonensis* (WHOM/BR/75/Josefa) were maintained in Schneider's insect medium (Sigma–Aldrich) supplemented with 10% FCS, at 26 °C. To infect human or murine macrophages, 4- to 5-day-old stationary promastigotes were washed twice, suspended in DMEM and added to cultures at a ratio of 6 parasites per macrophage. Infections were carried out for 4–6 h at 35 °C. Then, non-internalized parasites were washed out and fresh DMEM with 5% HS (human macrophages) or 10% FCS (murine cells) was replenished to the infected cultures. Next, *Leishmania*-infected cells were treated with different reagents, as follows, according to the specific protocol. Anti-human IL-27

(R&D Systems) and anti-human-IL-10 receptor (anti-IL-10R) neutralizing antibodies (Peprotech, USA) and the PKR inhibitor (PKRi, 527450, Merck), were used at 0.5 μg/mL, 2 μg/mL and 300 nM, respectively. Recombinant murine and human IL-27 (rmIL-27 and rhIL-27, respectively; R&D Systems, USA) were used at 1 ng/mL, unless otherwise stated. Infected cells were kept at standard culture conditions for 3–4 days and then washed, fixed with methanol and stained with Panótico (Laborclin; Pinhais, PR, Brazil). *Leishmania*-infected and non-infected macrophages, as well as the amount of parasites inside of the cells, were counted using light microscopy, and the infection index was calculated multiplying the percentage of infected macrophages by the average parasite number per cell, as described (Barreto-de-Souza et al., 2006).

Western blot analysis of PKR activation

To investigate whether IL-27 could activate PKR, 10^6 human primary macrophages were maintained in tissue culture flasks and exposed to rhIL-27 (1 ng/mL; R&D Systems) during different time points at 37 °C, 5% CO_2. To detect the total PKR levels and PKR activation, we carried out immunoblotting analysis with anti-PKR (Cell Signaling) or anti-Thr451 phospho-PKR antibodies [MBL International Corporation], respectively. The load of protein extracts applied to the immunoblots was normalized relative to the reaction with anti-GAPDH or anti-β-actin antibodies [Cell Signaling].

Quantitative real-time reverse transcriptase polymerase chain reaction

Following *L. amazonensis* infection for 4 h, the total RNA of peritoneal macrophages (10^6 cells) was extracted with an Invitrap® Spin Universal RNA Mini Kit (Invitek, Berlin, Germany) and a 1 μg aliquot of total RNA was reverse transcribed into first-strand cDNA with ImProm-II (Promega, Madison, WI) and an oligo(dT) 12–18 primers, according to the manufacturer's instructions. The following primers were used: IL-27p28 forward: 5′-CTCTGCTTCCTCGCTACCAC-3′ and reverse: 5′-GGGGCAGCTTCTTTT-CTTCT-3′; EBI3 forward: 5′-TCCAAGCTGCTCTTCCTGTCACTT-3′ and reverse: 5′-ATACCGAGAAGCATGGCATTGCAC-3′; GAPDH forward: 5′-TGCACCACCAACTGCTTAGC-3′ and reverse: 5′-GGCATGGACTGTGGTCATGAG-3′. Quantitative PCR (qPCR) was conducted using 95 °C (10 min) to denature DNA strands followed by 95 °C (30 s), then 60 °C (30 s) for 40 cycles. Amplicon specificity was carefully verified by the presence of a single melting temperature peak in dissociation curves. Real time quantitative RT-PCR (qRT-PCR) was performed using the Applied Biosystems StepOne™ detection system (Applied Biosystems, Foster City, CA) with GoTaq® qPCR Master Mix (Promega). qRT-PCR experimental data were normalized using GAPDH primers as an endogenous control. All expression ratios were computed via analysis of the relative gene expression $\Delta\Delta$Ct method through the StepOne software version 2.0 (Applied Biosystems).

IL-27 production measurement

RAW-bla and RAW-DN-PKR cells were cultured in 48-well plates, infected with *L. amazonensis* at a ratio of 6 parasites per cell and incubated for 4–6 h at 37 °C, 5% CO_2. Next, non-internalized parasites were removed by washing with PBS (pH 7.2) and infected cultures were maintained in DMEM supplemented with 10% FCS (Sigma) for 24 h at 37 °C, 5% CO_2. Then, IL-27 production was measured in the cell culture supernatants using a specific sandwich ELISA (R&D Systems), according to the manufacturer's instructions.

IL-27 treatment of Leishmania-infected mice

C57BL/6 mice (10 per group) were infected in the left hind footpad with 5×10^5 *L. amazonensis* promastigotes and, then, injected in the infected footpad with rmIL-27 (100 ng) or PBS (both in 10 μl volume) on days 2, 4 and 6 after infection. Lesion sizes were measured weekly with a digital caliper. At the indicated time-points, the mice were euthanized to determine the parasite burden by a limiting dilution assay, as previously described (Lima et al., 1997).

Statistical analyses

Results were analyzed using the Graphpad Prism v4.0 software (San Diego, CA) and are presented as the mean values ± standard error of the mean (SEM). Statistical analyses were performed using Student's *t* test, and two-way ANOVA for quantitative real-time PCR data. Comparisons between means were considered statistically significant when the *p* value was less than 0.05.

Results

L. amazonensis infection enhances macrophage expression of IL-27, which increases the parasite replication via IL-10 signaling

It has been reported that plasma levels of IL-27 are elevated in patients with visceral leishmaniasis and that PBMCs from *Leishmania braziliensis* infected individuals synthesize IL-27 mRNA *ex vivo* in response to soluble *Leishmania* antigens (Ansari et al., 2011; Novoa et al., 2011). Thus, we hypothesized that *L. amazonensis* infection could induce IL-27 expression. To test that assumption, we infected peritoneal macrophages with promastigotes of *L. amazonensis*, and 4 h later the total RNA was extracted to analyze the messenger RNA levels for the p28 and EBI3 subunits. As shown in Fig. 1A, *L. amazonensis*-infected macrophages exhibited increased levels of mRNA for both EBI3 and p28 units, which corroborated the notion that IL-27 expression is indeed augmented in macrophages infected with this parasite.

Because we observed that IL-27 is induced by *L. amazonensis*-infected macrophages, we next sought to investigate whether IL-27 could affect parasite proliferation. Initially, human macrophages infected with *L. amazonensis* promastigotes were exposed to different rhIL-27 concentrations. Three to four days after the cytokine addition we determined the cellular infection index, as described in Section "Materials and methods". As depicted in Fig. 1B, both tested concentrations of IL-27 (1 ng/mL and 5 ng/mL) were capable of increasing parasite growth in culture (1 ng/mL doubled the infection index). Of note, IL-27 significantly augmented the percentage of infected cells (the absolute values of intracellular parasite growth and parasite propagation in macrophages can be observed in Suppl. Fig. 1), suggesting that IL-27 not only increases *Leishmania* multiplication in macrophages but also drives parasite propagation in culture. The addition of anti-IL-27 neutralizing antibodies to the infected cell cultures significantly reduced the parasite multiplication (Fig. 1C), meaning that the naturally released IL-27 is indeed a favoring mediator of *L. amazonensis* growth in infected macrophages.

It is well known that IL-27 elicits IL-10 expression in macrophages and T cells in a variety of *in vivo* and *in vitro* models (Yoshida and Miyazaki, 2008; Yoshida et al., 2009; Freitas do Rosario et al., 2012). We have also previously demonstrated that PKR activation in the presence of *L. amazonensis* leads to IL-10 expression, which, in turn, favors parasite replication (Pereira et al., 2010). Thus, we addressed the contribution of IL-10 in the observed enhanced parasite multiplication triggered by IL-27. Accordingly, we treated human macrophages infected with *L. amazonensis* with

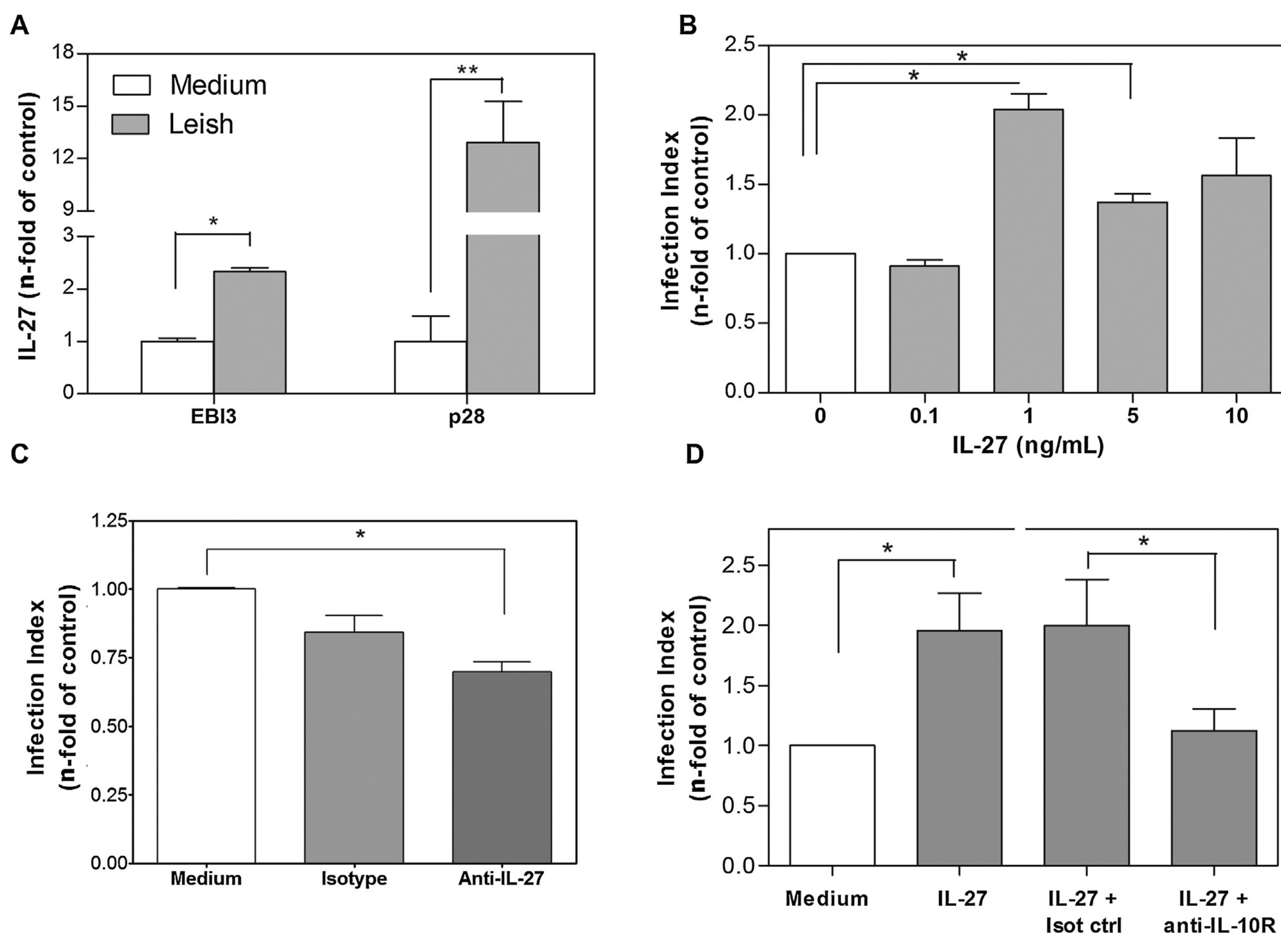

Fig. 1. *L. amazonensis* upregulates IL-27 expression, which increases parasite replication in macrophages mediated by IL-10. (A) Peritoneal macrophages from C57BL/6 mice were infected with *L. amazonensis* promastigotes. After 4 h total RNA was extracted and quantitative PCR was conducted for both EBI-3 and p28 subunits. Bars represent the means ± standard error of the mean (SEM) of 3 independent experiments. $^*p < 0.01$; $^{**}p < 0.005$. (B) Human macrophages were infected with *L. amazonensis* and, then, treated with rhIL-27, as indicated. Parasite growth was evaluated 3–4 days later by determining the infection index. Bars represent the means ± SEM of 4 independent experiments. $^*p < 0.01$. (C) Human macrophages were infected with *L. amazonensis* and treated with neutralizing antibodies to IL-27 (0.5 μg/mL) or the isotype control (Isot ctrl, 0.5 μg/mL). Parasite growth was evaluated as in (B). Bars represent the means ± SEM of 3 independent experiments. $^*p < 0.05$. (D) Human macrophages infected with *L. amazonensis* were treated with rhIL-27 (1 ng/mL) in the presence or not of neutralizing antibodies to the IL-10 receptor (anti-IL-10R, 2 μg/mL) or the isotype control (Isot ctrl, 2 μg/mL). Parasite growth was evaluated in (B). Bars represent the means ± SEM of 5 independent experiments. $^*p < 0.01$.

IL-27 in the presence of neutralizing antibodies to the IL-10 receptor (or an isotype antibody control). As we predicted, the IL-10 receptor blockage ablated the parasite load induced by IL-27 (Fig. 1D), indicating that the IL-27 enhancing effect on *L. amazonensis* growth was IL-10 dependent.

IL-27 favors L. amazonensis replication through PKR activation

Next, we investigated the signaling pathway involved in IL-27-favoring *L. amazonensis* proliferation in macrophages. Other authors have previously described that IL-27 is able to induce PKR gene expression in macrophages (Imamichi et al., 2008), and we have also revealed that PKR activation by the synthetic double-stranded RNA Poly(I:C) favors *L. amazonensis* growth by a mechanism dependent upon IL-10 secretion (Pereira et al., 2010). Therefore, we addressed whether IL-27 could activate PKR in macrophages and whether this activation would be relevant for the IL-27-mediated enhancement of parasite proliferation. For this purpose, we performed a blotting analysis of phospho-PKR threonine 451 (the active form of PKR). IL-27 treatment induced PKR activation in macrophages (as shown in Fig. 2A), without modulating the total PKR levels (Suppl. Fig. 2). Of note, we observed that IL-27 also induced PKR phosphorylation in PBMCs from normal donors during the same time period (data not shown).

In an attempt to evaluate the actual participation of PKR in the IL-27-driven enhancement of *L. amazonensis* growth, we used three different approaches: PKR inhibition with a specific synthetic molecule; expression of a dominant-negative PKR in macrophages; and macrophages from PKR genetically deficient mice. As depicted in Fig. 2B, treatment of macrophages with a PKR inhibitor (PKRi) almost completely abrogated the augmentation of *L. amazonensis* replication promoted by IL-27. Importantly, the spontaneous growth of *L. amazonensis* in human macrophages was not changed by the PKRi. Next, we compared the *L. amazonensis* growth in RAW 264.7 murine macrophages that were transfected with an empty vector (RAW-bla) or with a plasmid containing a gene that expresses a kinase-defective PKR (PKR-K296R), referred to as dominant-negative PKR (RAW-DN-PKR). As shown in Fig. 2C, dominant negative PKR expression abolished the effect of IL-27 when compared with control cells (RAW-bla), supporting the notion that PKR plays a pivotal role in the IL-27-induced *L. amazonensis* growth (the absolute values of intracellular parasite growth and parasite propagation in macrophages from control mice can be observed in Suppl. Fig. 1). To further confirm these findings, *L. amazonensis*-infected peritoneal macrophages from genetically deficient PKR (PKR-KO) mice, or from PKR sufficient animals (wild type, WT), were treated with rmIL-27. Whereas IL-27 more than doubled the *Leishmania* growth in WT mice, this interleukin was unable to

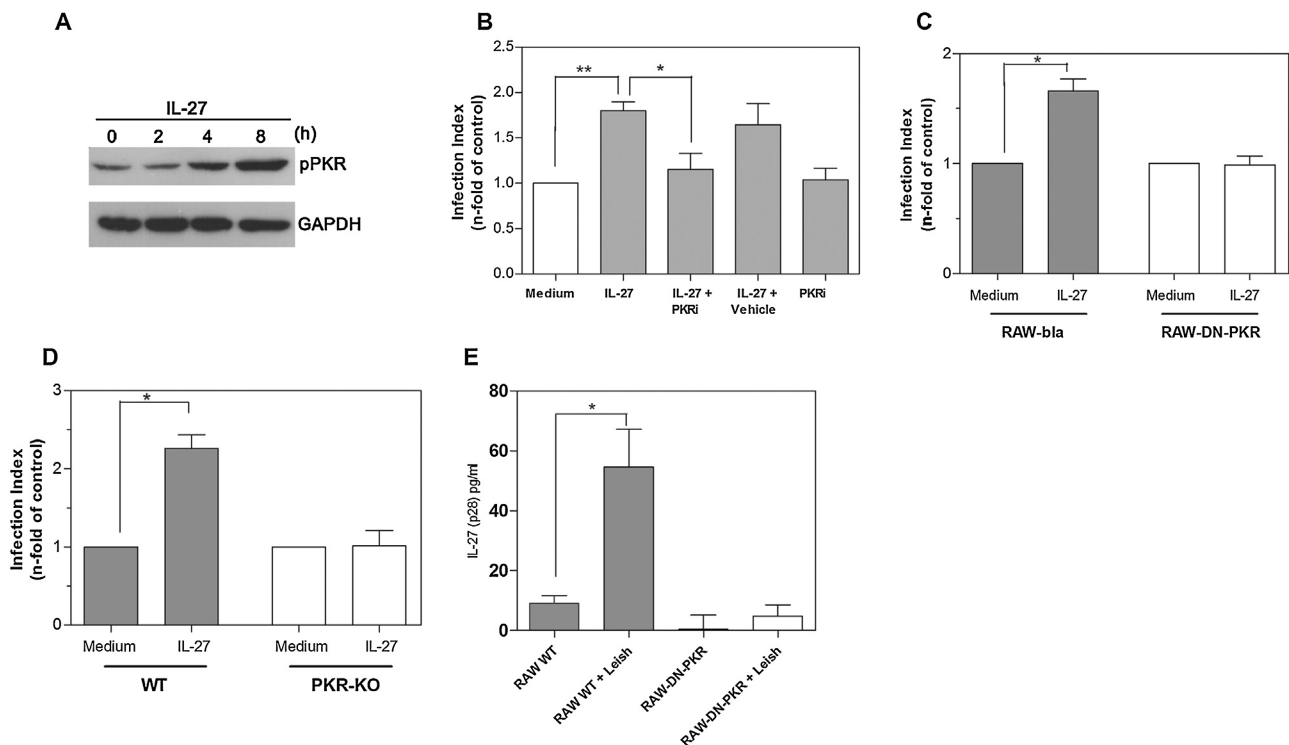

Fig. 2. IL-27 activates PKR, which is crucial to IL-27-mediated *Leishmania* augmentation. (A) Human macrophages were stimulated with rhIL-27 for different time periods, and total protein was then extracted from those cells. PKR activation was assessed by blotting analysis. One representative blotting is shown from 3 with similar results. (B) Human macrophages infected with *L. amazonensis* were stimulated with rhIL-27 (1 ng/mL) and treated or not with PKR inhibitor (PKRi, 300 nM) or with the vehicle of the PKRi. Parasite growth was assessed as in Fig. 1. Bars represent the means ± SEM of 5 independent experiments. $^*p < 0.05$; $^{**}p < 0.01$. (C) RAW 264.7 cells transfected with either an empty plasmid (RAW-bla) or with a kinase-defective PKR (RAW-DN-PKR) were infected with *L. amazonensis* and treated with rmIL-27 (1 ng/mL). Parasite replication was evaluated as in Fig. 1. Bars represent the means ± SEM of 3 independent experiments. $^*p < 0.05$. (D) Peritoneal macrophages from PKR-knockout (PKR-KO) or wild type (WT) mice were infected with *L. amazonensis* and treated with rmIL-27 (1 ng/mL). Parasite growth was assessed as stated before. Bars represent the means ± SEM of 4 independent experiments. $^*p < 0.05$. (E) RAW-WT and RAW-DN-PKR cells were infected with *L. amazonensis* and IL-27 production was measured by ELISA assay 24 h later. Bars represent the means ± SEM of 3 independent experiments. $^*p < 0.002$.

enhance parasite replication in the PKR-KO mouse macrophages (Fig. 2D), clearly demonstrating that IL-27 stimulated *L. amazonensis* multiplication through PKR signaling. These results also underscore that IL-27 favors the replication of *L. amazonensis* in murine macrophages, with a similar strengthening (2- to 3-fold increase) of that observed in human macrophages.

Because we observed that *L. amazonensis* infection increases IL-27 expression in macrophages, we next investigated the participation of PKR signaling in this mechanism. RAW-bla and RAW-DN-PKR cells were then infected with *L. amazonensis* and IL-27 production was evaluated with an ELISA assay. *L. amazonensis* increased IL-27 production only in RAW-bla cells (Fig. 2E), revealing the essential role of PKR signaling in the IL-27 augmented expression that was observed during *L. amazonensis* macrophage infection.

TLR2 engagement and IFN1 signaling are required for IL-27-induced expression by L. amazonensis

We have previously demonstrated that *L. amazonensis* activates the PKR/IFN1 axis via TLR2 (Vivarini et al., 2011), a critical pathway for the parasite replication in macrophages. To test whether *Leishmania*-induced IL-27 expression was dependent on TLR2 and IFN1 signaling, we carried out qRT-PCR analysis in macrophages derived either from $TLR2^{-/-}$ or $IFN1R^{-/-}$ mice. As predicted, the IL-27 levels were reduced in the infected macrophages derived from the knockout murine cells (Fig. 3). These data underline the relevance of TLR2 and IFN1 signaling for IL-27 expression and the consequent *Leishmania* amastigotes replication.

IL-27 increases L. amazonensis proliferation in vivo

To investigate the *in vivo* effect of IL-27 on *L. amazonensis* infection, mouse footpads were infected with *L. amazonensis* and then treated with rmIL-27, as described in Section “Materials and methods”. The IL-27 injections increased the footpad lesions in the second and third weeks following infection, when compared with the PBS control group (Fig. 4A). However, in the fourth week following infection the footpad lesion sizes were similar in both groups.

The parasite load was evaluated four weeks after infection. We found that the IL-27 inoculation in the infected mouse footpads strongly augmented the parasite load in the footpads and in the draining lymph nodes as well (Fig. 4B and C, respectively). Altogether, these data corroborate the *in vitro* analysis and unveil the ability of IL-27 to promote parasite growth in cutaneous leishmaniasis caused by *L. amazonensis*.

Discussion

The cytokine IL-27 plays many immune regulatory functions and holds prominent roles in a variety of inflammatory and infectious illnesses, including infections caused by protozoan parasites and viruses (Yoshida and Miyazaki, 2008; Yoshida et al., 2009; Sun et al., 2011; Greenwell-Wild et al., 2009; Jankowski et al., 2010). The majority of studies regarding the role of IL-27 in the context of parasitic diseases have focused on the development of the Th1 response, which usually promotes macrophage activation and weakens the replication of several intracellular pathogens.

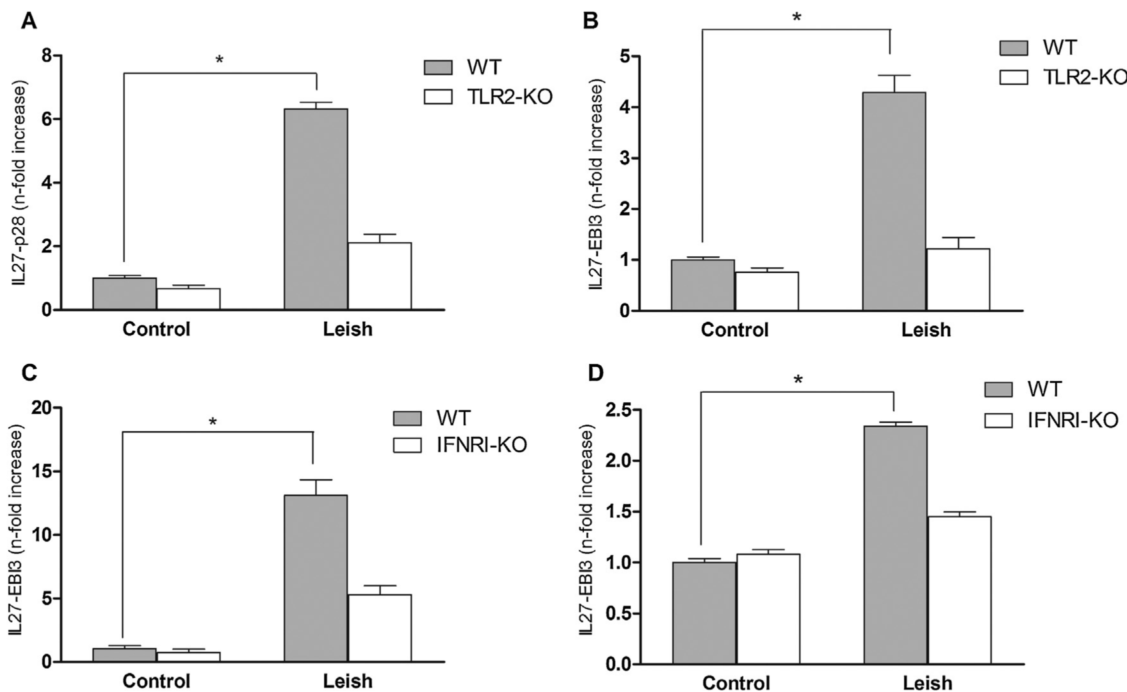

Fig. 3. *L. amazonensis*-induced expression of IL-27 requires TLR2 and IFN1 signaling. Peritoneal macrophages derived from TLR2-KO (A, B) or IFN1R-KO (C, D) mice were infected with *L. amazonensis* for 4 h, and IL-27 expression was assessed using quantitative real-time PCR. Bars represent the means ± SEM of 3 independent experiments. $^{*}p < 0.05$.

As already described, IL-27 is augmented in the plasma of patients affected by visceral forms of leishmaniasis (Ansari et al., 2011). Additionally, IL-27 synthesis is up-regulated in activated PBMCs from *L. braziliensis*-infected patients (Novoa et al., 2011), suggesting that IL-27 can be present in the milieu where *Leishmania*-infected macrophages reside. In this study, we tested whether IL-27 interferes with *L. amazonensis* replication, and found that this interleukin stimulates parasite growth in both, human and murine macrophages. Furthermore, we also observed that PKR and IL-10 are critical components of this process. We initially detected

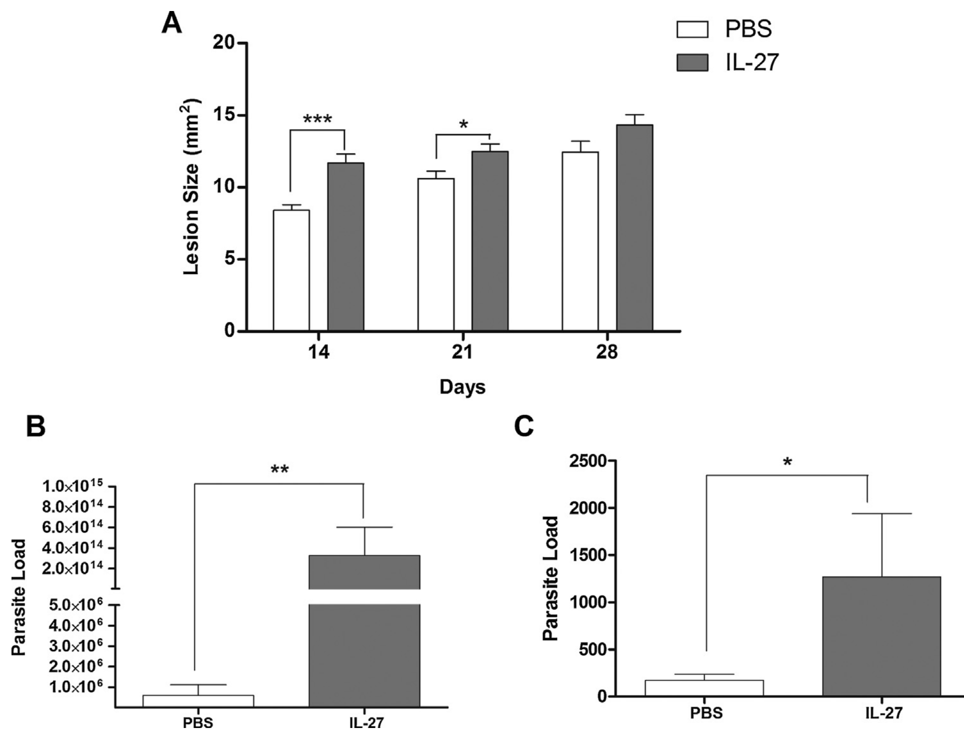

Fig. 4. IL-27 increases *L. amazonensis* replication *in vivo*. C57BL/6 mice (10 per group) were infected in the left hind footpad with 5×10^5 *L. amazonensis* promastigotes. Mouse groups were injected in the infected footpad with rmIL-27 (100 ng) or PBS (both in 10 μl volume) on days 2, 4 and 6 after infection. (A) Footpad lesion sizes were measured weekly with a digital caliper. After 28 days of infection, mice were euthanized, footpad (B) and draining lymph nodes (C) were excised and total parasite load was determined using a limiting dilution assay. $^{**}p < 0.01$; $^{*}p < 0.02$.

that the mRNA synthesis of both IL-27 subunits, EBI-3 and p28, was augmented in *Leishmania*-infected macrophages, a finding that suggests that infected macrophages could contribute to the enhanced IL-27 plasma levels in *Leishmania*-harboring patients.

We observed that IL-27 treatment increased *L. amazonensis* replication in macrophages and that IL-27 injection in the *Leishmania*-infected mouse footpads increased the lesion size and augmented the parasite load in the mouse footpads and lymph nodes, indicating that this cytokine exerted a local and a systemic effect on parasite growth and propagation. Because murine dendritic cells can synthesize IL-27 a few hours after *L. donovani* inoculation (Maroof and Kaye, 2008), we think that sentinel tissue cells produce this cytokine soon after encountering *Leishmania*, which favors its growth in resident macrophages.

The host protection from excessive tissue and organ damage secondary to inflammation or infections is an important feature of the IL-27 modulatory roles (Yoshida and Miyazaki, 2008; Yoshida et al., 2009). In this context, it is possible that IL-27 not only supports parasite establishment and growth but also protects the host from damage by unrestrained inflammatory responses. As the *Leishmania* infection progresses, the generation of Th2 responses can contribute to parasite replication and maintenance within the infected mammal. In accordance with the above mentioned dual role of IL-27 in *Leishmania* infection are our *in vivo* results, which show augmented parasite loads due to IL-27 treatment, while the lesion sizes were comparable in both groups after the fourth week of infection. Interestingly, a recent report showed that IL-27 enhanced the survival of the Gram-negative bacterium *Burkholderia pseudomallei* by depressing microbicidal cellular mechanisms (Rinchai et al., 2012). This underlies, together with our findings, the possible significant ability of IL-27 to modulate infections caused by a diversity of different pathogens.

The addition of exogenous IFN1 exhibits an ambiguous role in the control of *L. major* infection (Pereira et al., 2010; Khouri et al., 2009; Bogdan et al., 2004). However, recent data indicate that IFN1 enhances *L. amazonensis* infection due to the expression of superoxide-dismutase 1 (SOD1) (Pereira et al., 2010; Khouri et al., 2009). We have previously demonstrated that TLR2 engagement is required for the augmented expression of IFN1 and PKR in macrophages infected with *L. amazonensis* (Pereira et al., 2010). Moreover, it has also been shown that TLR2 is important for IL-27 expression (Kim et al., 2011). Accordingly, we described that *L. amazonensis* replicate poorly inside macrophages from TLR2-KO mice (Vivarini et al., 2011). In the present work we show that IL-27 expression is severely reduced in infected macrophages from TLR2-KO or IFN1R-KO mice. These data corroborate the notion that TLR2 and IFN1 are important for *Leishmania*-induced IL-27 expression and may contribute to the IL-27 effect on *L. amazonensis* intracellular survival.

To understand the biochemical pathway involved in the IL-27-mediated *L. amazonensis* growth augmentation, we analyzed the role of PKR in this phenomenon, and found that this kinase is activated by IL-27, likewise reported by other authors (Liu et al., 2012). We previously described that *L. amazonensis* is favored when PKR is activated (Pereira et al., 2010), and detected a pronounced PKR activation in the biopsies of patients with severe *L. amazonensis* infections (Vivarini et al., 2011), suggesting that the PKR signaling pathway is active in patients suffering of leishmaniasis. In fact, *L. amazonensis per se* is able to activate PKR in infected macrophages (Vivarini et al., 2011). The molecules that induce PKR activation in the context of leishmaniasis have not been identified yet, but we believe that IL-27 and *L. amazonensis*-derived molecules can contribute to this biochemical phenomenon *in vivo*. When analyzed all together, our data, which were obtained from infected macrophages from PKR genetically deficient mice, from cells expressing a kinase-defective PKR, and from human macrophages treated with a PKR inhibitor, reveal that the activation of this kinase is central to *Leishmania* growth triggered by IL-27. These results corroborate our previous studies that placed PKR as a regulator of *Leishmania* infection (Pereira et al., 2010), and suggest that other PKR activators might also have similar effects on *Leishmania* infections. Thus, based on our present and previous results (Pereira et al., 2010; Vivarini et al., 2011), as well as on reports from other authors (Kim, et al., 2011), we believe that the *Leishmania*-induced TLR2 ligation in infected macrophages leads to PKR activation, which, in turn, promotes IL-27 synthesis. Once produced, this cytokine triggers new cycles of PKR activation, which favors parasite growth. Taking this signaling pathway into account, the inactivation of PKR would provoke a deficiency in IL-27 production and, ultimately, diminish *Leishmania* replication.

Other studies have underscored the importance of the IL-27 signaling pathway in the generation of a protective Th1 response after experimental infection of WSX-1 knockout mice with *L. major* (Artis et al., 2004; Yoshida et al., 2001). It is important to stress that, whereas PKR signaling favors *L. amazonensis* infection, the activation of this kinase may lead to a decrease of *L. major* replication (Pereira et al., 2010). Overall, these observations highlight the complexity of the PKR-dependent response in the orchestration of escape mechanisms triggered by distinct *Leishmania* species.

In conclusion, our results place IL-27 as an important molecule in *L. amazonensis* replication in mammalian host cells through PKR activation and IL-10 signaling, revealing a novel feature of the modulatory activities of this cytokine. Taken together, our results regarding IL-27-driven PKR activation and its effect on parasite replication warrant additional studies concerning the ability of IL-27 to modulate the growth of other pathogenic protozoa.

Conflict of interest

The authors declare that they do not have any conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2014.11.006.

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